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Apoptosis Gene Expression in Glioblastoma Multiforme Tumor Cells Treated with Cisplatin and Gamma Irradiation

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By

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Capstone Approval Page

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ABSTRACT (100 – 200 WORDS):

Glioblastoma multiforme (GBM) is the most prevalent and aggressive malignant primary brain tumor in humans. Once diagnosed, patient survival times range from three months if left untreated to a 12 percent survival rate at two years with palliative treatment. Death is usually a result of cerebral edema and increased intracranial pressure associated with a necrotic mass. Treatment of GBM is very difficult due to the vulnerability of healthy brain tissue to conventional treatments and the restricted ability of the brain to repair itself, as well as the difficulties associated with administering chemotherapeutic agents across the blood brain barrier. Treatment with gamma irradiation and chemotherapy concurrently has been shown to significantly improve patient survival rates when compared to tumor resection alone (Patwardhan et. al., 2004). The expression of apoptosis genes in response to treatment with cisplatin or a combination cisplatin and gamma irradiation in two GBM cell lines, U87 and U251, was investigated by quantitative real time polymerase chain reaction (qPCR). Ideally, up regulation of genes associated with pro-apoptotic pathways and down regulation of genes associated with anti-apoptotic pathways would increase levels of programmed cell death by apoptosis, as opposed to necrosis. This may result in decreased cerebral edema and pressure, thereby increasing patient survival rates.

Introduction

Glioblastoma Multiforme (GBM) is the most prevalent and aggressive malignant primary brain tumor in humans (CBTRUS, 2011). Once diagnosed, the patient's prognosis is grim. Due to the aggressive nature of GBM, survival times range from three months if untreated, to an average of 1-2 years with palliative treatment. Treated patients have only a twelve percent survival rate at two years and median survival of only 15 months (CBTRUS, 2011). Death is usually a result of cerebral edema and increased intracranial pressure associated with a necrotic mass. Treatment of GBM is very difficult for several reasons. First, the GBM cells are very resistant to conventional therapies and healthy brain tissue is vulnerable to these therapies. This can have a detrimental effect on the brain, given the restricted ability to repair itself (Lawson et al., 2007). Another consideration in treating GBM with chemotherapeutic agents is the decreased efficiency or inability of large molecules to cross the blood brain barrier and effectively target the tumor cells.

Treatment of GBM cells by gamma irradiation and chemotherapy concurrently has been shown to significantly improve patient survival rates when compared to tumor resection alone (Patwardhan et al., 2004). The effects of gene regulation on patient survival are unclear at this time. However, apoptosis array analysis may provide insight into how GBM cells die by apoptosis after undergoing various treatments. Ideally, the treatments would up regulate pro-apoptotic genes and down regulate anti-apoptotic genes, promoting increased levels of apoptosis and reduced levels of necroptosis (Castellino et al., 2007). Programmed cell death as a result of apoptosis may lead to increased patient survival by decreasing edema and intracranial pressure associated with necrotic cell death.

Two GBM cell lines were used in this study. The U87 cell line is wild type for the p53 gene, an essential tumor suppressor gene capable of regulating cell cycle. The U251 cell line is defective in the p53 gene (van Meir et al., 1994). Cells from each tumor line have been cultured, treated, collected and have undergone quantitative real time polymerase chain reaction using 96-well Human RT^2 Apoptosis qPCR Arrays (Qiagen) to analyze and determine the regulation of 84 genes associated with apoptosis as a result of treatment with cisplatin or a combination of cisplatin and gamma irradiation.

Materials and Methods

Human tissue samples. U87 and U251 GBM cell lines were obtained from Dr. David Grdina and Dr. Jeff Murley at the University of Chicago and Dr. Howard Fine at the National Institutes of Health. The cell lines were originally obtained from a stage three tumor in a 44 year old Caucasian female. The tumor expressed both U87 tumor cells and U251 tumor cells expressing a mutation of the p53 gene, causing it to be present but dysfunctional (van Meir et al., 1994).

Cell Culture and Collection. GBM cells were maintained in monolayer culture in DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum and penicillin/streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The following cell doubling times were obtained using similar conditions to those described above: U87 cells exhibited doubling times of 29.8 hours, whereas U251 cells doubled in quantity slightly faster, within 25.9 hours (Ke et al., 2000). Growth medium was discarded from T75 flask after a maximum of three days and replenished with 10mL fresh 37°C DMEM:F12. Cells were subcultured using 0.0625% trypsin in Ca^{++} and Mg^{++} free Hank's balanced saline solution (HBSS)

and 0.02% EDTA for 6 minutes for U251 cells and 3 minutes for U87 cells (Gupta et al.,1997; Yasui et al., 2008). Cells were then collected into centrifuge tubes, pelleted by centrifugation (1000 rpm, 2 minutes, 12°C) and resuspended in fresh medium for counting. The number of cells obtained was calculated by viewing 10μL of cell suspension on a hemocytometer under an inverted microscope. The hemocytometer count was then divided by four and multiplied by 10,000 to determine the number of cells per milliliter in the sample. Approximately 150,000 cells were replated onto the T75 flask to be maintained in a monolayer culture. T75 flasks were trypsinized a maximum of three times before being discarded, at which time cells were plated onto a fresh T75 flask.

Serial dilutions were performed in order to obtain required concentrations for seeding onto 100mm and 60mm Petri plates. See Table 8 for the plating concentrations used in the experimental trials. Growth medium was collected into 50mL centrifuge tubes from the Petri plates prior to trypsinization in order to collect floating cells. Cells were trypsinized as previously described and collected into the same 50mL centrifuge tubes. Two milliliters of fresh medium were added to the Petri plates, which were then carefully scraped with rubber policemen to avoid overlapping scraped areas. Dislodged cells remained intact for collection. The vials were pelleted by centrifugation, medium was removed and cell pellets were resuspended in fresh medium to give approximate concentrations of less than 1×10^6 cells per milliliter. Exact concentrations were determined by hemocytometer count. Cells were divided and collected in 15mL centrifuge tubes to obtain approximately $1\text{-}2 \times 10^6$ cells per tube. Samples were pelleted by centrifugation, medium was removed and cells were resuspended and washed in 1mL PBS. The cell suspensions were then transferred to 1.5mL microfuge tubes

using a pipetman and microfuged at 16,000 x g to repellet. PBS was carefully pipetted off of the pellets. Labeled microfuge tubes were then placed into the -80°C freezer for at least 10 minutes prior to RNA isolations.

Cisplatin Treatment and Collection. Cells from each tumor line were subcultured onto 100mm and 60mm Petri plates containing 10mL and 5mL growth medium, respectively. Several dilutions of cells were plated onto the Petri plates according to the length and type of treatment subjected to. Tables 1 and 2 show the plating dilutions and collection totals for the experimental conditions in both U87 and U251 cell lines.

Two experimental conditions were established using cisplatin. The first condition applied only a 1μM cisplatin-growth medium solution for a period of either three days or seven days of continuous exposure. The second experimental condition also included continuous exposure to a 1μM cisplatin-growth medium solution, as well as 10 Gray gamma irradiation, as described below.

The 1μM cisplatin-growth medium solution was prepared first as a 2mM cisplatin-PBS solution by the addition of 5mg cisplatin to 8.3mL PBS solution. The 1μM solution was prepared by the addition of 50μL of the 2mM cisplatin-PBS solution per 100mL of growth medium. The original growth medium in the plates designated for treatment with cisplatin was discarded and 10mL of the 1μM cisplatin-growth medium solution were added to each of the 100mm Petri plates. The cells on the 60mm Petri plates were treated with 5mL of the 1μM cisplatin-growth medium solution.

The Petri plates were then returned to the incubator. After three or seven days the cisplatin-growth medium was collected into 50mL centrifuge tubes prior to trypsinization to collect floating cells. The plates were then trypsinized as previously described for experimental

conditions. After pelleting by centrifugation, the cisplatin-medium solution was decanted into the cisplatin waste container and the pellet was resuspended in fresh growth medium to determine cell count. The cells were again separated into separate 15mL centrifuge tubes to obtain 1-2 x 10^6 cells per vial, pelleted by centrifugation and resuspended in 1mL PBS. Once washed with PBS, the cells were transferred into 1.5mL microfuge tubes, pelleted at 16,000 x g and PBS was carefully pipetted off. Microfuge tubes were labeled and stored in -80°C freezer prior to RNA isolations.

Gamma Irradiation and Collection. U251 cells were subcultured onto 60mm Petri plates with 5mL growth medium at varying concentrations according to Tables 1 and 2. Treatments with gamma irradiation were only conducted in conjunction with 1μM cisplatin treatments.

Gamma irradiation was delivered by subjecting three stacked 60mm Petri plates to a Cesium-137 gamma source for five minutes and sixteen seconds. (Dose rate 1.9 Gray/minute) The cells were treated with gamma irradiation three hours after receiving the 5mL of 1μM cisplatin-growth medium solution. The cells were trypsinized and collected, as described above, after three days or seven days of continuous exposure to 1μM cisplatin-growth medium solution. The cells were labeled and stored in -80°C freezer until RNA isolations were performed.

Table 1: U251 Plating Concentrations and Collection Totals

	3 Day Collections			7 Day Collections		
Treatment	Cells Plated	Collection Date	Total Cells	Cells Plated	Collection Date	Total Cells
-CIS/-RAD	100,000 x1	20111202	1.43 x 10	20,000 x2	20111206	3.74 x 10
+CIS/-RAD	200,000 x1	20111202	1.46 x 10	20,000 x3	20111206	1.07 x 10
+CIS/+RAD	150,000 x2	20111202	1.02 x 10	30,000 x3	20111206	1.38 x 10

Table 2: U87 Plating Concentrations and Collection Totals

Treatment	3 Day Collections			7 Day Collections		
	Cells Plated	Collection Date	Total Cells	Cells Plated	Collection Date	Total Cells
-CIS/-RAD	50,000 x2 100,000 x2	20120206	1.71×10^6	20,000 x4 40,000 x2	20120210	3.28×10^6
+CIS/-RAD	100,000 x6	20120206	1.57×10^6	20,000 x4 40,000 x2	20120210	2.35×10^6
+CIS/+RAD	150,000 x1 200,000 x5 250,000 x1	20120206	1.16×10^6	150,000 x3 200,000 x5 250,000 x1	20120210	1.40×10^6

RNA Isolation. RNA isolations procedures were performed using an Ambion PureLink RNA Mini Kit (Catalog No. 12183-018A, lot 844860) according to the protocols for samples of less than 5×10^6 cells. 1mL of Lysis solution was pipetted into one 1.5mL microfuge tube and labeled for each sample collected. 10µL 2-mercaptoethanol was mixed thoroughly with the lysis solution to promote denaturation of RNases and other proteins. 0.6mL of the lysis buffer solution (with 2-mercaptoethanol) was transferred into the respectively labeled 1.5mL microfuge tubes containing cell pellets. A sterile pestle was used to grind the cells for 30 seconds. The homogenate was then transferred to a separate labeled 1.5mL microfuge tube using a pipetman. The microfuge tube and pestle were rinsed using one volume (0.6 mL) of 70% ethanol and added to the cell homogenate using a pipetman. The samples in the microfuge tubes were mixed using a labquake for 5 minutes at room temperature to obtain a homogeneous mixture.

600µL of the mixture was then transferred into spin cartridges containing a silica resin containing a proprietary combination of salts, which selectively binds RNA. The addition of 70%

ethanol enhances RNA binding to the silica. Spin cartridges were inserted into collection tubes and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow through was discarded and the spin cartridge was reinserted into the same collection tube. The remaining 600µL of the sample was then transferred into the spin cartridge and it was again centrifuged as described before. Once the entire sample had been processed, 600µL of Wash Buffer I (proprietary mixture) was added to the spin cartridge to remove proteins and contaminants from the silica resin. The spin cartridge was centrifuged at 12,000 x g for 15 seconds at room temperature. The flow through was discarded along with the collection tube. The spin cartridge was then placed into a new collection tube. 500µL of Wash Buffer II (proprietary mixture) containing 100% ethanol was added to spin cartridge to remove remaining salts from the silica resin. The spin cartridge was centrifuged at 12,000 x g for 15 seconds at room temperature. The flow through was discarded and the spin cartridge was reinserted into the same collection tube for one additional wash with 500µL of Wash Buffer II. The samples were centrifuged as described before, after which the flow through was discarded and the spin cartridges were reinserted into the same collection tubes.

The cartridges were centrifuged for 2 minutes at 12,000 x g to dry the membrane and remove the remaining ethanol. The flow through and collection tubes were discarded. The spin cartridges were placed into new collection tubes and centrifuged an additional 2 minutes at 12,000 x g. Flow through and collection tubes were discarded and the spin cartridges were inserted into recovery tubes labeled with the date of isolation, cell line, treatment type and treatment length. 50 µL of RNase-free water (0.05% Diethylpyrocarbonate (DEPC)) was pipetted directly onto the center of the spin cartridge membrane to rehydrate the silica resin. Samples were incubated at room temperature for 1 minute and then centrifuged for 2 minutes at

21,000 x g at room temperature to elute RNA from membrane into the recovery tube. Elution was repeated once with an additional 50 µL of RNase-free water. A total sample of 100 µL was obtained in each recovery tube. Samples were then stored in the -20°F freezer until gel electrophoresis was conducted.

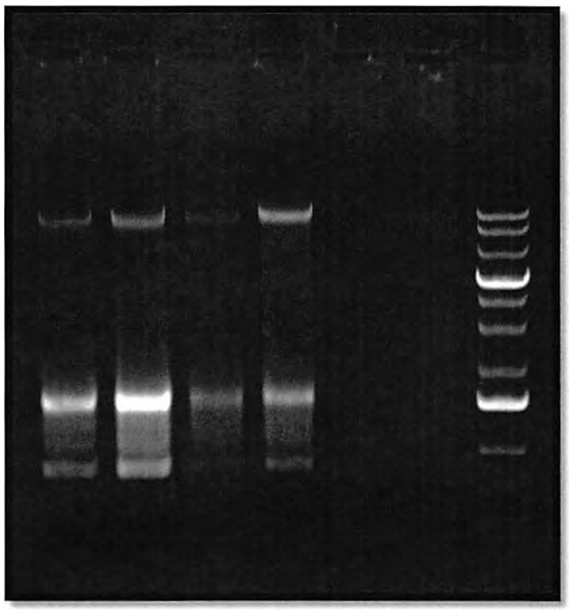
Gel Electrophoresis. A 1% Agarose mini Tris-Acetate-EDTA (TAE) gel solution was prepared in a graduated bottle containing 96mL of distilled water. 4mL of 25X TAE Buffer (0.04 M Tris base, 0.04 M Acetate, 0.001 M EDTA), 1g agarose powder and 30µL ethidium bromide were added to the distilled water and swirled gently. The cap was replaced and loosely sealed. The bottle was then heated at 30% power in the microwave for approximately 2 minutes while observing for first signs of boiling. Once the contents were completely dissolved, the bottle was swirled to ensure solubilization of agarose. The contents were allowed to cool slightly in room temperature water bath, ensuring that hazing and solidification were not observed. The casting tray was prepared and the liquid gel solution was poured slowly into the tray until the solution level was approximately halfway from the top of the tape. The comb was then inserted into the end of the tray and aligned so that all the wells were centered. The gel was allowed to cool, undisturbed, for approximately 30 minutes, until completely solidified.

RNA electrophoresis samples were prepared in 1.5mL microfuge tubes, each containing 10µL gel loading buffer (GLB) (6% sucrose, 0.1% bromophenol blue) solution. 5µL of each of the RNA samples were added to the corresponding 1.5mL microfuge tubes and mixed thoroughly using a pipetman. 5µL of Fermentas O'geneRuler™ 1kb Plus DNA Ladder (0.1µg/µL) was added to a separate 1.5mL microfuge tube containing 10µL GLB. The prepared gel was submerged in 1% TAE buffer solution in the electrophoresis chamber, aligning the wells along the edge of the plate at the anode of the chamber. 15µL of each of the samples were then carefully loaded into

a designated lane of the gel using a pipetman. The electrophoresis chamber was then covered and the electrodes were connected. The voltage was set to 150 Volts delivered continuously over the course of 25 minutes. The gel was then removed from the electrophoresis chamber for image capture and analysis.

Image Capture and Analysis. The gels were placed into the ultraviolet chamber for image capture using Kodak 1D imaging software. Three separate image files were obtained under the UV light source. The first image captured was standard UV image without saturation, saved as a bitmap (.bip) file. The second was the same captured image exported to a tagged image file format (.tif). Finally, the third image collected contained saturation levels, obtained using the software and was saved as a separate bitmap (.bip) file.

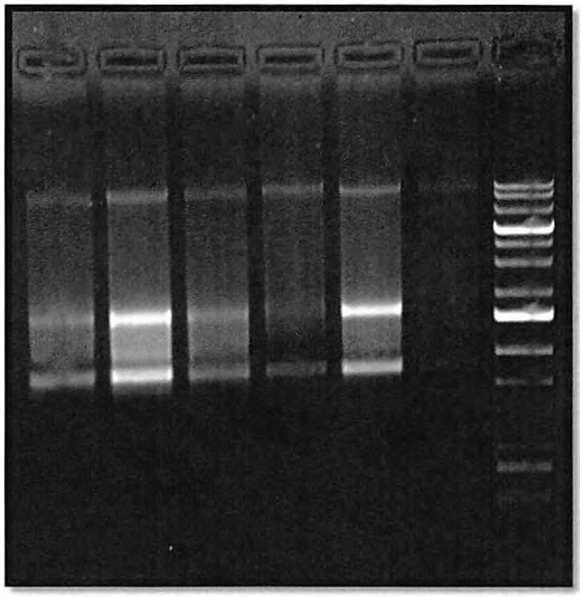
Figure 1: First U251 RNA Electrophoresis Gel #2040



From Left:
Lane 1: 5µL 3d -CIS/-RAD U251 RNA; 15.44 ng RNA/µL
Lane 2: 5µL 3d +CIS/-RAD U251 RNA; 25.65 ng RNA/µL
Lane 3: 5µL 3d +CIS/+RAD U251 RNA; 5.45 ng RNA/µL
Lane 4: 5µL 7d -CIS/-RAD U251 RNA; 10.64 ng RNA/µL
Lane 5: 5µL 7d +CIS/-RAD U251 RNA; 4.44 ng RNA/µL
Lane 6: 5µL 7d +CIS/+RAD U251 RNA; 2.33 ng RNA/µL
Lane 7: 5µL O'gene 1kb Plus DNA Ruler; 80 ng RNA/0.5µg

Analysis of the images was performed using the Kodak 1D imaging software. Images were uploaded into the software and were straightened and aligned. The bands were correlated to the individual lanes of the gel using imaging software. The bands surrounding the 1.5kb RNA band from the DNA ladder were selected to determine net

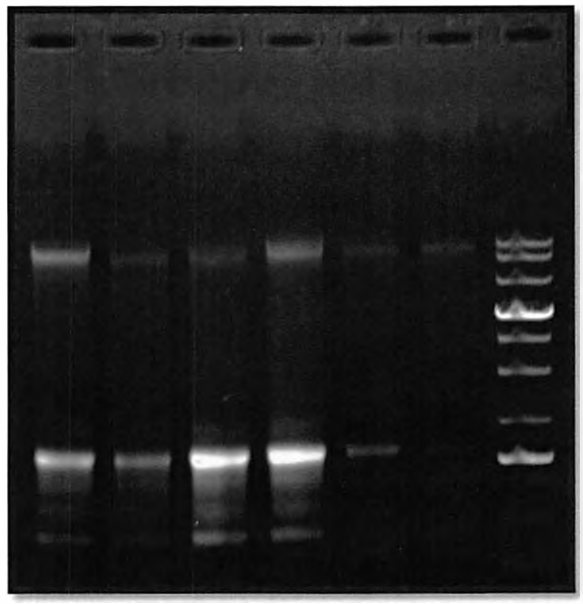
Figure 2: Second U251 RNA Electrophoresis Gel #2041



From Left:

Lane 1: 5µL 3d -CIS/-RAD U251 RNA; 7.41 ng RNA/µL
Lane 2: 5µL 3d +CIS/-RAD U251 RNA; 20.02 ng RNA/µL
Lane 3: 5µL 3d +CIS/+RAD U251 RNA; 6.05 ng RNA/µL
Lane 4: 5µL 7d -CIS/-RAD U251 RNA; 4.22 ng RNA/µL
Lane 5: 5µL 7d +CIS/-RAD U251 RNA; 19.47 ng RNA/µL
Lane 6: 5µL 7d +CIS/+RAD U251 RNA; 2.28 ng RNA/µL
Lane 7: 5µL O'gene 1kb Plus DNA Ruler; 80 ng RNA/0.5µg

Figure 3: U87 RNA Electrophoresis Gel #2043



From Left:

Lane 1: 5µL 3d -CIS/-RAD U87 RNA; 14.53 ng RNA/µL
Lane 2: 5µL 3d +CIS/-RAD U87 RNA; 6.78 ng RNA/µL
Lane 3: 5µL 3d +CIS/+RAD U87 RNA; 25.12 ng RNA/µL
Lane 4: 5µL 7d -CIS/-RAD U87 RNA; 25.57 ng RNA/µL
Lane 5: 5µL 7d +CIS/-RAD U87 RNA; 5.96 ng RNA/µL
Lane 6: 5µL 7d +CIS/+RAD U87 RNA; 3.00 ng RNA/µL
Lane 7: 5µL O'gene 1kb Plus DNA Ruler; 80 ng RNA/0.5µg

intensities. The software determined the net intensities for each of the bands above and below the 1.5kb RNA band standard. The net intensities were recorded for the bands in individual lanes. The net intensity of the 1.5kb RNA band of the O'gene Plus 1kb DNA Ruler was also obtained and used as the standard. According to Fermentas' O'Gene 1kb Plus Ruler the 1.5kb band of the ruler contains 80ng RNA/0.5µg (0.1µg/1µL). Analysis of RNA content (ng RNA/µL) for each of the samples was calculated by adding both net intensities for each lane, dividing by the net intensity of the 1.5kb band of the DNA ruler, multiplying by the amount of DNA at the 1.5kb band of the DNA ruler (e.g. 80ng) and finally the dividing by the amount of the RNA sample present in each of the lane wells (e.g. 5µL).

Sample calculation: Gel 2041 Lane 1 20120220 3d U87 RNA -CIS/-RAD

The net intensities of the bands located above and below the 1.5kb standard of the DNA ruler were added together: 112445 + 195599 = 308044. The sum of the net intensities of each lane was divided by the net intensity of the 1.5kb band in lane 7: 308044 ÷ 145266 = 2.12. The result was multiplied by 80ng RNA per microliter and divided by the number of microliters of sample used: 2.12 x 80ng RNA ÷ 5µL = 33.93 ng RNA per microliter.

Table 3: U87 Electrophoresis Gel #2043 Analysis Data

Lane	Contents	Net Intensities	Concentration (ng/uL)
1	5µL 20120220 U87 RNA 3d -CIS -RAD	112445, 195599	33.93
2	5µL 20120220 U87 RNA 3d +CIS -RAD	50434, 11118	6.78
3	5µL 20120220 U87 RNA 3d +CIS +RAD	195259, 32815	25.12
4	5µL 20120220 U87 RNA 7d -CIS -RAD	202113, 30022	25.57
5	5µL 20120220 U87 RNA 7d +CIS -RAD	43058, 11060	5.96
6	5µL 20120220 U87 RNA 7d +CIS +RAD	21203, 6052	3.00
7	5µL O'gene 1.5kb plus DNA Ruler	145266	80

Table 4: U251 Electrophoresis Gels #2040 & #2041 Analysis Data

Lane	Contents	Net Intensities (Gel #2040)	Concentration (ng/uL)	Net Intensities (Gel #2041)	Concentration (ng/uL)	Average Concentration (ng/uL)
1	5µL U251 RNA 3d -CIS -RAD	82896, 38380	15.44	14907, 34891	7.41	11.43
2	5µL U251 RNA 3d +CIS -RAD	144956, 56476	25.65	70033, 64578	20.02	22.84
3	5µL U251 RNA 3d +CIS +RAD	24674, 18142	5.45	13424, 27210	6.05	5.75
4	5µL U251 RNA 7d -CIS -RAD	53255, 30346	10.64	7605, 20758	4.22	7.43
5	5µL U251 RNA 7d +CIS -RAD	25871, 9010	4.44	70875, 59951	19.47	11.96
6	5µL U251 RNA 7d +CIS +RAD	10837, 7496	2.33	2902, 12427	2.28	2.31
7	5µL O'gene 1.5kb plus DNA Ruler	125658	80	107531	80	80

DNase Treatment. The RNA isolates were subjected to DNase treatments in order to remove any remaining double stranded DNA from the samples prior to cDNA synthesis and qPCR analysis. Reactions were performed in 0.2mL PCR vials and required 9µL of RQ1 DNase 10x Reaction Buffer (Promega M198A), 1µL of RQ1 RNase-free DNase Enzyme (Promega M610A) and 10µL of RQ1 Stop Solution, added after incubation (Promega M199A). 80µL of RNA isolate were added for a total of 100µL per sample reaction. Tables 5 and 6 show the reaction setup and amount of RNA used for each of the reactions in the U87 and U251 experiments, respectively.

Table 5: U87 RNA DNase Treatments

Tube	Contents	RNA ng/uL	RNA Solution (uL)	Total RNA (ng)	DNase Buffer (uL)	DNase Enzyme (uL)	Stop Solution (uL)	Total Sample (uL)
1	20120220 U87 RNA 3d -CIS -RAD	33.93	80	2714.4	9	1	10	100
2	20120220 U87 RNA 3d +CIS -RAD	6.78	80	542.4	9	1	10	100
3	20120220 U87 RNA 3d +CIS +RAD	25.12	80	2009.6	9	1	10	100
4	20120220 U87 RNA 7d -CIS -RAD	25.57	80	2045.6	9	1	10	100
5	20120220 U87 RNA 7d +CIS -RAD	5.96	80	476.8	9	1	10	100
6	20120220 U87 RNA 7d +CIS +RAD	3.00	80	240.0	9	1	10	100

Table 6: U251 RNA DNase Treatments

Tube	Contents	RNA ng/uL (Avg.)	RNA Solution (uL)	Total RNA (ng)	DNase Buffer (uL)	DNase Enzyme (uL)	Stop Solution (uL)	Total Sample (uL)
1	20120123 U251 RNA 3d -CIS -RAD	11.43	80	914.4	9	1	10	100
2	20120123 U251 RNA 3d +CIS -RAD	22.84	80	1827.2	9	1	10	100
3	20120123 U251 RNA 3d +CIS +RAD	5.75	80	460.0	9	1	10	100
4	20120123 U251 RNA 7d -CIS -RAD	7.43	80	594.4	9	1	10	100
5	20120123 U251 RNA 7d +CIS -RAD	11.96	80	956.8	9	1	10	100
6	20120123 U251 RNA 7d +CIS +RAD	2.31	80	184.8	9	1	10	100

The contents of each vial were added in the following order: 80µL RNA sample, 9µL RQ1 DNase 10x Reaction Buffer and 1µL RQ1 RNase-free DNase Enzyme. The vials were placed into the thermocycler and incubated at 37°C for 2 hours. After the incubation 10µL RQ1 DNase Stop Solution were added, bringing the total contents of each vial to 100µL. The vials were returned to the thermocycler for a 10 minute incubation at 65°C to inactivate the DNase enzymes. Following the incubation, the vials were centrifuged at 21,000 times gravity for approximately 10 seconds. The entire contents (100µL) of each vial were transferred using a pipetman into 1.5mL microfuge tubes labeled with the date, treatment length and type, cell line and corresponding tube number (e.g. 20120227 U251 3d +/- DNase 2). 10µL 3M sodium acetate from prepared stock was added to each vial, creating a 0.3M sodium acetate concentration in solution. Two volumes (220µL) of 95% ethanol were added to each tube and mixed gently. The tubes were placed into the -20°C freezer overnight for precipitation. After overnight precipitation, the tubes were warmed to 37°C and centrifuged for 15 minutes at 21,000 times gravity to pellet. The supernatant was carefully decanted, ensuring the pellet remained in the microfuge tubes. The microfuge tubes containing the pellets were inverted, allowing any excess supernatant to be removed and dried. The pellets were resuspended and washed in 0.5mL 70% ethanol on the vortex for several seconds, then repelleted by centrifugation at 21,000 times gravity for 5 minutes. Supernatant was again carefully decanted, ensuring the pellets remained in the microfuge tubes, which were inverted again for several minutes to dry. The tubes were then placed into the Savant Speed Vac (Model SC110, Serial 191117) for 10 minutes or until completely dried. Pellets were resuspended in 12µL DEP treated water and vortexed for several seconds, then stored in the -20°C freezer until cDNA synthesis and –RT control reactions were run.

cDNA Synthesis Reactions. The DNase treated RNA samples were removed from the -20°C freezer and warmed to 37°C. For each cell line 8µL of each DNase sample were transferred into corresponding 0.2mL PCR tubes labeled 1, 2, 3, 4, 5 or 6. One microliter of Oligonucleotide 09413 decamer, 2µL of a 100x dilution of 061511 RNA3 from stock and 2.5µL of DEP treated water were added to each PCR vial for a total solution volume of 13.5µL. The PCR tubes were placed into the thermocycler for 5 minutes at 70°C. The PCR tubes were placed on ice immediately after the incubation period. While incubating, the common components for each of the reactions plus one additional aliquot were combined. Common components included 0.5µL per reaction of RNAsin (Promega), 5µL per reaction of 2.5mM dNTPs (Takara Bio Inc.) and 5µL per reaction of 5x Reaction Buffer (Promega). The common components were gently mixed and 10.5µL were pipetted into each of the labeled 0.2mL PCR tubes. One microliter (200u/µL) of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) was added to each PCR tube and gently mixed. Each tube containing a volume of 25µL mixture was placed into the thermocycler and run using the program "cdnapromega42", an automated incubation cycle, which incubated the samples at 42°C for 1 hour, 95°C for 5 minutes and then holds the samples at 10°C until the cDNA could be diluted and frozen. The entire 25µL of cDNA from each of the PCR tubes were transferred into corresponding 1.5mL microfuge tubes. 225µL of DEP treated water were added to each of the microfuge tubes, producing a 10x dilution. Tubes were labeled with dilution factor, date, cell line, treatment length and type and cDNA reaction number. (e.g. 10x Diln 20120301 U251 3d +/- cDNA 2) and stored in -20°C freezer until the qPCR positive controls were run.

Negative Reverse Transcriptase Control. A negative reverse transcriptase (RT) control was prepared from each of the DNase samples. The purpose of the negative RT control is to determine if any contaminating genomic DNA is present after the DNase treatments were conducted. The negative RT controls should show high Ct values when run in qPCR compared to the Ct values of the cDNA obtained from the reverse transcriptase reactions.

To determine the dilution factor required for the negative RT controls, the overall dilution factor with respect to the 10X cDNA was calculated. The 25µL cDNA solution contained 8 µL of DNase treated RNA, creating an original dilution of 3.125:1 (25 µL/8 µL), which was further diluted 10 times by the addition of 225 µL of DEP treated water. The total cDNA dilution factor was 31.25 µL of solution per 1 µL of DNase treated RNA added. In order to standardize the negative RT controls to this dilution factor, 2 µL of DNase treated RNA was added to labeled 1.5mL microfuge tubes for each of the treatments from both cell lines. To obtain the 31.25:1 dilution, 62.50 µL of solution were required for each reaction. Each tube already contained 2 µL of RNA and with the addition of 60.50 µL of DEP water, a dilution of 31.25:1 was obtained. The tubes were labeled 31.25X Diluition, date, cell line and corresponding treatment type. The negative RT controls were stored in the -20°C freezer until the positive control PCR reactions were run.

Quantitative Real-Time PCR.

Positive Control Reactions. Positive control reactions were conducted using cDNA and negative reverse transcription DNase controls and served multiple purposes. First, reactions having undergone reverse transcription successfully will have produced cDNA, detectable by PCR in relatively fewer cycles than a negative control. Second, the negative control reactions serve to indicate that the reverse transcription was successful and that the DNase treatments

were also successful in denaturing any double stranded contaminating genomic DNA that would produce false PCR results.

The positive PCR reactions utilized a primer mixture (Yasui Mix A) containing designated primers 07564 and 07565, specific to 18S rRNA sequences. Two 0.2mL PCR tubes were used for each treatment type, one for cDNA and the other for the negative RT control. A total of 24 reactions were conducted with the reaction setups and results listed in tables 7 and 8 below. The inclusion of 12.5µL of SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich #S4438) was used for quantification during qPCR and contained the heat stable Taq (*Thermus aquaticus*) polymerase (0.05 units/µL).

Table 7: U251 Positive Control qPCR Reactions

Well Number	Primer Mix YA (µL)	Sample Contents (Cisplatin/Gamma) (µL)	SYBR (µL)	Ct (dRn)
121257	9.5	3µL 20120301 10X dilution U251 cDNA 1 3d (–/–)	12.5	18.07
121258	9.5	3µL 20120301 31.25X dilution U251 Dnase 1 3d (–/–)	12.5	No Ct
121259	9.5	3µL 20120301 10X dilution U251 cDNA 2 3d (+/–)	12.5	17.37
121260	9.5	3µL 20120301 31.25X dilution U251 Dnase 2 3d (+/–)	12.5	38.5
121261	9.5	3µL 20120301 10X dilution U251 cDNA 3 3d (+/+)	12.5	18.7
121262	9.5	3µL 20120301 31.25X dilution U251 DNase 3 3d (+/+)	12.5	No Ct
121263	9.5	3µL 20120301 10X dilution U251 cDNA 4 7d (–/–)	12.5	15.74
121264	9.5	3µL 20120301 31.25X dilution U251 Dnase 4 7d (–/–)	12.5	No Ct
121265	9.5	3µL 20120301 10X dilution U251 cDNA 5 7d (+/–)	12.5	17.27
121266	9.5	3µL 20120301 31.25X dilution U251 Dnase 5 7d (+/–)	12.5	No Ct
121267	9.5	3µL 20120301 10X dilution U251 cDNA 6 7d (+/+)	12.5	20.46
121268	9.5	3µL 20120301 31.25X dilution U251 DNase 6 7d (+/+)	12.5	No Ct

Table 8: U87 Positive Control qPCR Reactions

Well Number	Primer Mix YA (µL)	Sample Contents (Cisplatin/Gamma) (µL)	SYBR (µL)	Ct (dRn)
121861	9.5	3µL 20120316 10X dilution U87 cDNA 1 3d (–/–)	12.5	15.68
121862	9.5	3µL 20120316 31.25X dilution U87 Dnase 1 3d (–/–)	12.5	38.91
121863	9.5	3µL 20120316 10X dilution U87 cDNA 2 3d (+/–)	12.5	15.06
121864	9.5	3µL 20120316 31.25X dilution U87 Dnase 2 3d (+/–)	12.5	38.67
121865	9.5	3µL 20120316 10X dilution U87 cDNA 3 3d (+/+)	12.5	11.41
121866	9.5	3µL 20120316 31.25X dilution U87 DNase 3 3d (+/+)	12.5	No Ct
121867	9.5	3µL 20120316 10X dilution U87 cDNA 4 7d (–/–)	12.5	13.52
121868	9.5	3µL 20120316 31.25X dilution U87 Dnase 4 7d (–/–)	12.5	No Ct
121869	9.5	3µL 20120316 10X dilution U87 cDNA 5 7d (+/–)	12.5	18.93
121870	9.5	3µL 20120316 31.25X dilution U87 Dnase 5 7d (+/–)	12.5	38.70
121871	9.5	3µL 20120316 10X dilution U87 cDNA 6 7d (+/+)	12.5	19.42
121872	9.5	3µL 20120316 31.25X dilution U87 DNase 6 7d (+/+)	12.5	38.42

The contents were added in the order of primer mixture, cDNA or RNA sample and then SYBR Green. The tubes were centrifuged at 1000 times gravity for 10 seconds, vortexed for 10 seconds to mix thoroughly and centrifuged again at 1000 times gravity for 10 seconds. The PCR tubes were loaded into the PCR thermocycler and run for 40 cycles. Each cycle consisted of a 95°C dissociation for 30 seconds, a 60°C annealing phase for 30 seconds and a 72°C extension phase for 60 seconds, with a final extension of 60 seconds and a temperature hold of 4°C until stored in the -20°C freezer.

Apoptosis Arrays. In order to identify the effects of cisplatin and combination cisplatin and gamma irradiation treatments on the genes associated with apoptosis in U87 and U251 GBM cell lines, Human RT^2 Apoptosis Arrays (Qiagen PAHS-012A) were used. The arrays contained 96 wells, each with designated primer sequences specific to a targeted gene. Eighty four of the

wells contained primers specific to apoptosis related genes, while the other 12 wells contained primer sequences for housekeeping genes, positive PCR reactions and reverse transcription controls.

The apoptosis arrays were stored in the -20°C freezer until the reactions were to be conducted to ensure stability of the primers. Upon use, the array was brought to room temperature, centrifuged, unopened, at 1000 times gravity for 15 seconds. The core components for the reaction setup were added together into two separate 1.5mL microfuge tubes each containing enough for 55 (25µL) reactions. The common components per tube included 572µL (10.43µL/reaction) of DEP water, 692µL (12.5µL/reaction) of SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich #S4438), 100µL (1.82µL/reaction) of cDNA sample from one of the treatment types of a given cell line and 13.75µL (0.25µL/reaction) of R4526 Reference Dye (Sigma-Aldrich). Each well of the array received a total of 25µL of the core mixture. After filling each column of eight wells in the array a strip tube cover was placed atop of the wells to avoid contamination. Half of the arrays (48 wells) were prepared using one of the core component mixtures, and the second mixture was used to prepare the second halves of the arrays. Once loaded, the arrays were centrifuged at 1000 times gravity for 15 seconds, vortexed for 15 seconds to mix and centrifuged again. The arrays were loaded into the qPCR thermocycler and underwent 40 cycles of 95°C dissociations for 30 seconds, a 60°C annealing phases for 30 seconds and a 72°C extension phases for 60 seconds, with a final extensions of 60 seconds and a temperature hold of 4°C.

Results

Positive Control PCR Reactions

The purpose of this experiment was to determine the effectiveness of the reverse transcriptase in preparation of the cDNA samples from the DNase treated RNA samples. The 31.25x dilutions of the DNase treated RNAs were used as negative reverse transcription controls in order to determine if any contaminating genomic DNA was present after treatments with DNase. The cDNA samples showed much lower threshold (Ct) values than the DNase RNA samples, indicating that there was successful reverse transcription of the mRNA isolated from the U87 and U251 cells. The negative reverse transcription controls showed much higher threshold values due to lower concentrations of the desired template, present only as contaminating genomic DNA. For actual threshold (Ct) values, see tables 7 and 8 above.

Apoptosis Array PCR Reactions

Six apoptosis arrays were conducted for each cell lineage. Two arrays for cisplatin only treatment, two for cisplatin and gamma irradiation concurrent treatment and two for untreated controls. Each of the arrays from the treatment groups corresponded to either three or seven day treatments.

Tables 9 and 10 provide the fold regulations for each of the treatment types compared to the untreated controls at either three or seven days. The data includes the 84 apoptosis related genes and 5 housekeeping genes used for standardization. Genes appearing in red indicate pro-apoptotic genes and those appearing in black represent anti-apoptotic genes. Wells H1-H5 consist of the housekeeping genes and have no bearing on regulation of apoptosis

in the cells. Figures 4-11 depict graphical representations of the fold value regulations observed when comparing the treated cells to the corresponding untreated controls at three and seven days.

Conclusions

Analysis of the results provided no definitive conclusions on whether or not treatment with cisplatin alone or concurrent treatment with cisplatin and gamma irradiation had any effect on the regulation of apoptosis. There are several intricate cellular pathways that require regulation at multiple steps allowing for apoptosis to proceed. Here we focused on five pro-apoptotic genes specific to the DNA damage pathway leading to apoptosis. The five genes, ABL1, CIDEA, CIDEB, TP53 and TP73, were found to have undergone slight (under three-fold) up and down regulation. U251 cells showed over a 4.5 fold increase in CIDEA at the seven day treatment length with cisplatin and gamma irradiation. U87 cells showed significant gene regulation differences in the TP53 and TP73 tumor suppressor genes. In both treatments for the seven day treatment period, the TP53 gene was down regulated nearly 3.5 fold. TP53 is an essential pro-apoptotic tumor suppressor protein and its down regulation is may indicate that cell death is occurring in a manner other than apoptosis, through either necrosis or autophagy. TP73, a tumor suppressor protein belonging to the TP53 family of tumor suppressor proteins, was initially down regulated approximately 5.5 fold in the U87 three day combination treatment. However, both U87 seven day treatments show significant up regulation of the TP73 gene. There was approximately a fifty fold increase in the expression levels of TP73 in these cells. Over expression of TP73 in medullablastoma tumors has been shown to increase

levels of apoptotic cell death in similar studies (Castellino et al., 2007). Continued analysis into the expression levels of the other genes associated with apoptosis pathways may provide more insight into the mechanisms of cell death that the cells underwent as a result of the treatments.

Table 9: U251 PCR Fold Regulations

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U251 3 Day +Cisplatin -Gamma vs 3 Day Control	U251 3 Day +Cisplatin +Gamma vs 3 Day Control	U251 7 Day +Cisplatin -Gamma vs 7 Day Control	U251 7 Day +Cisplatin +Gamma vs 7 Day Control
A1	ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	+	-1.28	-1.51	1.55	1.27
A2	AKT1	V-akt murine thymoma viral oncogene homolog 1	-	-1.30	-1.34	-1.99	-1.13
A3	APAF1	Apoptotic peptidase activating factor 1	+	-1.07	-1.07	1.29	-1.09
A4	BAD	BCL2-associated agonist of cell death	+	-1.18	1.86	-1.04	-1.28
A5	BAG1	BCL2-associated athanogene	-	-1.71	-1.49	1.30	1.64
A6	BAG3	BCL2-associated athanogene 3	-	-1.17	-7.22	1.02	1.41
A7	BAG4	BCL2-associated athanogene 4	-	1.17	1.20	1.17	1.37
A8	BAK1	BCL2-antagonist/killer 1	+	1.01	1.24	2.46	2.17
A9	BAX	BCL2-associated X protein	+	-1.38	-1.01	-1.24	1.13
A10	BCL10	B-cell CLL/lymphoma 10	+	1.27	2.15	-1.02	1.68
A11	BCL2	B-cell CLL/lymphoma 2	-	1.92	-3.49	-1.05	13.83
A12	BCL2A1	BCL2-related protein A1	-	2.84	5.93	1.09	1.69
B1	BCL2L1	BCL2-like 1	-	1.09	1.25	-2.20	1.27
B2	BCL2L10	BCL2-like 10 (apoptosis facilitator)	-	-2.47	1.01	2.30	4.69
B3	BCL2L11	BCL2-like 11 (apoptosis facilitator)	+	1.86	6.40	-2.39	-1.49
B4	BCL2L2	BCL2-like 2	-	1.22	1.12	1.88	2.10
B5	BCLAF1	BCL2-associated transcription factor 1	+	-1.00	-1.71	1.16	-1.30
B6	BFAR	Bifunctional apoptosis regulator	-	-1.30	-1.54	1.72	1.74
B7	BID	BH3 interacting domain death agonist	+	-1.14	1.45	-1.02	2.68
B8	BIK	BCL2-interacting killer (apoptosis inducing)	+	-2.47	1.01	2.23	5.86
B9	NAIP	NRL family, apoptosis inhibitory protein	-	1.20	-1.12	2.99	2.69
B10	BIRC2	Baculoviral IAP repeat containing 2	+	1.45	1.41	1.30	1.97
B11	BIRC3	Baculoviral IAP repeat containing 3	-	6.62	25.41	-2.91	10.27
B12	XIAP	X-linked inhibitor of apoptosis	-	3.70	1.03	-5.43	-1.67
C1	BIRC6	Baculoviral IAP repeat containing 6	-	1.25	2.34	1.23	1.39
C2	BIRC8	Baculoviral IAP repeat containing 8	-	-3.44	-1.39	2.30	7.31
C3	BNIP1	BCL2-adenovirus E1B 19kDa interacting protein 1	+	-1.24	1.70	1.11	-1.05
C4	BNIP2	BCL2-adenovirus E1B 19kDa interacting protein 2	-	1.06	-1.17	1.43	1.45
C5	BNIP3	BCL2-adenovirus E1B 19kDa interacting protein 3	+	1.01	1.45	1.49	1.27
C6	BNIP3L	BCL2-adenovirus E1B 19kDa interacting protein 3-like	+	-1.28	-1.29	4.26	2.33
C7	BRAF	V-raf murine sarcoma viral oncogene homolog B1	-	1.21	1.27	1.58	1.43
C8	NOD1	Nucleotide-binding oligomerization domain containing 1	+	1.08	1.92	1.61	1.39
C9	CARD6	Caspase recruitment domain family, member 6	+	-1.77	1.28	1.40	1.64
C10	CARD8	Caspase recruitment domain family, member 8	+	-1.51	-8.65	-1.49	-1.09
C11	CASP1	Capase 1, apoptosis-related cysteine peptidase	+	-1.05	1.41	2.51	1.32

Table 9: U251 PCR Fold Regulations (Cont.)

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U251 3 Day +Cisplatin -Gamma vs 3 Day Control	U251 3 Day +Cisplatin +Gamma vs 3 Day Control	U251 7 Day +Cisplatin -Gamma vs 7 Day Control	U251 7 Day +Cisplatin +Gamma vs 7 Day Control
C12	CASP10	Caspase 10 , apoptosis-related cysteine peptidase	+	-2.02	1.01	2.30	4.69
D1	CASP14	Caspase 14 , apoptosis-related cysteine peptidase	+	-1.76	-1.45	-2.75	3.71
D2	CASP2	Caspase 2 , apoptosis-related cysteine peptidase	+	-1.28	-1.04	1.05	-1.58
D3	CASP3	Caspase 3 , apoptosis-related cysteine peptidase	+	1.18	1.25	-1.13	-1.40
D4	CASP4	Caspase 4 , apoptosis-related cysteine peptidase	+	-1.30	-1.02	2.55	2.77
D5	CASP5	Caspase 5 , apoptosis-related cysteine peptidase	+	-2.47	1.01	2.30	4.69
D6	CASP6	Caspase 6 , apoptosis-related cysteine peptidase	+	-1.54	-1.50	2.62	-1.27
D7	CASP7	Caspase 7 , apoptosis-related cysteine peptidase	+	-1.22	1.26	2.03	1.06
D8	CASP8	Caspase 8 , apoptosis-related cysteine peptidase	+	1.04	1.49	1.30	1.62
D9	CASP9	Caspase 9 , apoptosis-related cysteine peptidase	+	-1.17	1.17	2.68	2.79
D10	CD40	CD40 molecule, TNF receptor superfamily member 5	+	-2.47	1.01	2.30	4.69
D11	CD40LG	CD40 ligand	+	-2.47	1.33	2.30	17.27
D12	CFLAR	CASP8 and FADD-like apoptosis regulator	-	-1.38	1.37	1.39	1.07
E1	CIDEA	Cell death-inducing DFFA-like effector a	+	-2.47	1.01	2.30	4.69
E2	CIDEB	Cell death-inducing DFFA-like effector b	+	-1.92	-1.07	-1.04	1.18
E3	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	+	1.02	1.77	1.31	1.02
E4	DAPK1	Death-associated protein kinase 1	+	-1.01	1.61	6.41	1.55
E5	DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	+	-1.32	-1.52	1.87	1.49
E6	FADD	Fas (TNFRSF6)-associated via death domain	+	1.05	1.50	-1.49	-1.06
E7	FAS	Fas (TNF receptor superfamily, member 6)	+	1.20	1.31	1.36	1.92
E8	FASLG	Fas ligand (TNF receptor superfamily, member 6)	+	-2.47	1.59	2.30	4.69
E9	GADD45A	Growth arrest and DNA-damage-inducible, alpha	+	2.56	25.95	-5.31	6.28
E10	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	+	-2.21	9.37	-1.10	19.70
E11	IGF1R	Insulin-like growth factor 1 receptor	-	1.56	2.07	-1.26	-1.43
E12	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	+	-1.74	1.71	1.29	4.20
F1	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	+	-1.22	1.22	1.57	1.60
F2	MCL1	Myeloid cell leukemia sequence 1 (BCL-related)	-	1.12	2.78	-1.29	-1.11
F3	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	-	-1.43	-1.24	8.63	5.70
F4	PYCARD	PYD and CARD domain containing	+	-1.84	1.94	4.66	23.10
F5	RIPK2	Receptor-interacting serine-threonine kinase 2	-	1.31	1.66	1.04	2.73
F6	TNF	Tumor necrosis factor	+	-3.23	1.30	2.30	51.98
F7	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	+	-2.47	1.01	2.30	4.69
F8	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	+	1.49	2.00	1.42	1.71
F9	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	+	-2.47	1.68	2.30	9.00
F10	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	+	-1.33	1.38	-56.49	-1.39

Table 9: U251 PCR Fold Regulations (Cont.)

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U251 3 Day +Cisplatin -Gamma vs 3 Day Control	U251 3 Day +Cisplatin +Gamma vs 3 Day Control	U251 7 Day +Cisplatin -Gamma vs 7 Day Control	U251 7 Day +Cisplatin +Gamma vs 7 Day Control
F11	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	+	-1.01	-1.66	1.68	1.80
F12	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	+	1.45	2.73	2.43	2.41
G1	CD27	CD27 molecule	-	-1.83	-1.05	1.78	8.17
G2	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	+	1.11	10.91	-4.82	12.13
G3	TNFRSF10	Tumor necrosis factor (ligand) superfamily, member 10	+	2.08	2.02	1.17	3.25
G4	CD70	CD70 molecule	+	1.19	1.41	-1.53	-1.33
G5	TNFRSF8	Tumor necrosis factor (ligand) superfamily, member 8	+	4.46	1.01	2.64	13.83
G6	TP53	Tumor protein p53	+	-1.40	-1.43	1.68	1.27
G7	TP538P2	Tumor protein p53 binding protein, 2	+	-1.04	1.08	2.03	1.54
G8	TP73	Tumor protein p73	+	-2.16	-1.68	1.45	2.77
G9	TRADD	TNFRSF1A-associated via death domain	+	-1.18	1.44	1.64	1.78
G10	TRAF2	TNF receptor-associated factor 2	-	-1.06	1.79	-1.00	1.44
G11	TRAF3	TNF receptor-associated factor 3	+	1.11	1.64	1.01	1.42
G12	TRAF4	TNF receptor-associated factor 4	-	-1.22	1.15	1.83	1.21
H01	B2M	Beta-2 microglobulin	N/A	1.37	1.40	1.51	2.35
H02	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	N/A	1.20	-1.38	-1.33	1.04
H03	RPL13A	60S ribosomal protein L13a	N/A	-1.26	1.26	-1.41	-1.47
H04	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	N/A	-1.31	-1.29	1.25	-1.65
H05	ACTB	Beta-actin	N/A	-1.51	-2.48	1.62	1.17

Table 10: U87 PCR Fold Regulations

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U87 3 Day +Cisplatin -Gamma vs 3 Day Control	U87 3 Day +Cisplatin +Gamma vs 3 Day Control	U87 7 Day +Cisplatin -Gamma vs 7 Day Control	U87 7 Day +Cisplatin +Gamma vs 7 Day Control
A1	ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	+	1.24	1.35	-2.34	-1.86
A2	AKT1	V-akt murine thymoma viral oncogene homolog 1	-	1.03	1.21	1.02	-1.02
A3	APAF1	Apoptotic peptidase activating factor 1	+	1.36	1.41	-1.79	1.11
A4	BAD	BCL2-associated agonist of cell death	+	-1.12	1.13	1.77	1.64
A5	BAG1	BCL2-associated athanogene	-	-1.02	-1.79	-2.63	1.10
A6	BAG3	BCL2-associated athanogene 3	-	1.09	1.36	-2.90	-2.92
A7	BAG4	BCL2-associated athanogene 4	-	1.25	1.03	-1.41	-1.11
A8	BAK1	BCL2-antagonist/killer 1	+	-2.07	-1.22	-1.12	-1.11
A9	BAX	BCL2-associated X protein	+	-1.11	-1.47	2.07	1.71
A10	BCL10	B-cell CLL/lymphoma 10	+	1.05	1.28	-1.53	-1.72
A11	BCL2	B-cell CLL/lymphoma 2	-	6.73	-2.48	42.30	13.41
A12	BCL2A1	BCL2-related protein A1	-	1.28	1.16	3.25	21.78
B1	BCL2L1	BCL2-like 1	-	1.73	1.58	1.76	1.97
B2	BCL2L10	BCL2-like 10 (apoptosis facilitator)	-	1.24	1.22	12.66	7.65
B3	BCL2L11	BCL2-like 11 (apoptosis facilitator)	+	1.87	-1.88	1.22	4.61
B4	BCL2L2	BCL2-like 2	-	1.13	2.11	-1.98	1.51
B5	BCLAF1	BCL2-associated transcription factor 1	+	1.08	-1.19	-3.38	-2.24
B6	BFAR	Bifunctional apoptosis regulator	-	1.09	1.21	-2.31	-2.06
B7	BID	BH3 interacting domain death agonist	+	1.13	1.15	-1.34	1.36
B8	BIK	BCL2-interacting killer (apoptosis inducing)	+	-1.75	-1.21	10.80	6.52
B9	NAIP	NRL family, apoptosis inhibitory protein	-	-1.01	1.44	-2.90	-1.13
B10	BIRC2	Baculoviral IAP repeat containing 2	+	1.16	-1.00	-1.15	2.20
B11	BIRC3	Baculoviral IAP repeat containing 3	-	1.83	1.81	1.08	1.74
B12	XIAP	X-linked inhibitor of apoptosis	-	4.11	-1.07	41.43	-1.19
C1	BIRC6	Baculoviral IAP repeat containing 6	-	3.29	-1.42	4.54	3.06
C2	BIRC8	Baculoviral IAP repeat containing 8	-	-2.04	-12.15	44.40	27.76
C3	BNIP1	BCL2-adenovirus E1B 19kDa interacting protein 1	+	-1.16	-1.24	-1.17	-1.98
C4	BNIP2	BCL2-adenovirus E1B 19kDa interacting protein 2	-	1.06	1.18	-1.82	-1.42
C5	BNIP3	BCL2-adenovirus E1B 19kDa interacting protein 3	+	1.51	3.22	1.07	2.42
C6	BNIP3L	BCL2-adenovirus E1B 19kDa interacting protein 3-like	+	1.15	3.03	-2.56	-1.44
C7	BRAF	V-raf murine sarcoma viral oncogene homolog B1	-	-1.12	1.38	-3.40	-2.82
C8	NOD1	Nucleotide-binding oligomerization domain containing 1	+	-1.31	-1.06	-1.27	1.53
C9	CARD6	Caspase recruitment domain family, member 6	+	1.49	1.22	-1.39	1.09
C10	CARD8	Caspase recruitment domain family, member 8	+	1.52	1.92	-1.82	-3.02
C11	CASP1	Capase 1, apoptosis-related cysteine peptidase	+	1.14	1.08	-1.15	-1.16

Table 10: U87 PCR Fold Regulations (Cont.)

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U87 3 Day +Cisplatin -Gamma vs 3 Day Control	U87 3 Day +Cisplatin +Gamma vs 3 Day Control	U87 7 Day +Cisplatin -Gamma vs 7 Day Control	U87 7 Day +Cisplatin +Gamma vs 7 Day Control
C12	CASP10	Caspase 10 , apoptosis-related cysteine peptidase	+	1.46	3.52	-7.00	-1.19
D1	CASP14	Caspase 14 , apoptosis-related cysteine peptidase	+	1.38	-10.80	186.43	48.67
D2	CASP2	Caspase 2 , apoptosis-related cysteine peptidase	+	-1.03	-1.03	-1.29	-1.75
D3	CASP3	Caspase 3 , apoptosis-related cysteine peptidase	+	1.61	-1.17	1.13	1.46
D4	CASP4	Caspase 4 , apoptosis-related cysteine peptidase	+	-1.02	1.56	-2.01	-1.91
D5	CASP5	Caspase 5 , apoptosis-related cysteine peptidase	+	-1.75	1.48	6.60	3.99
D6	CASP6	Caspase 6 , apoptosis-related cysteine peptidase	+	1.01	-1.11	1.07	-1.83
D7	CASP7	Caspase 7 , apoptosis-related cysteine peptidase	+	-1.01	-1.17	-1.06	1.18
D8	CASP8	Caspase 8 , apoptosis-related cysteine peptidase	+	-1.28	1.01	-1.64	-1.37
D9	CASP9	Caspase 9 , apoptosis-related cysteine peptidase	+	-1.11	-1.00	-1.03	-1.03
D10	CD40	CD40 molecule, TNF receptor superfamily member 5	+	-1.69	1.60	-1.01	-1.14
D11	CD40LG	CD40 ligand	+	-1.75	-2.15	6.42	3.88
D12	CFLAR	CASP8 and FADD-like apoptosis regulator	-	-1.04	1.16	-1.15	1.63
E1	CIDEA	Cell death-inducing DFFA-like effector a	+	-2.19	1.10	-1.63	-2.70
E2	CIDEB	Cell death-inducing DFFA-like effector b	+	-1.09	-1.11	-1.93	1.76
E3	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	+	1.12	-1.57	1.28	1.83
E4	DAPK1	Death-associated protein kinase 1	+	2.45	1.34	21.00	36.89
E5	DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	+	-1.06	1.66	-3.60	-3.77
E6	FADD	Fas (TNFRSF6)-associated via death domain	+	1.10	-1.70	2.25	1.57
E7	FAS	Fas (TNF receptor superfamily, member 6)	+	1.43	2.26	-2.80	-1.72
E8	FASLG	Fas ligand (TNF receptor superfamily, member 6)	+	-2.16	-2.05	19.46	11.75
E9	GADD45A	Growth arrest and DNA-damage-inducible, alpha	+	-1.03	1.54	2.13	4.33
E10	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	+	-3.81	2.20	1.35	-1.83
E11	IGF1R	Insulin-like growth factor 1 receptor	-	1.42	-1.95	1.23	4.24
E12	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	+	-3.48	-7.85	28.49	17.21
F1	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	+	1.02	-1.09	1.15	-1.20
F2	MCL1	Myeloid cell leukemia sequence 1 (BCL-related)	-	-1.01	-2.42	4.15	7.75
F3	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	-	-1.20	2.28	-2.05	-1.51
F4	PYCARD	PYD and CARD domain containing	+	-1.10	-2.59	2.30	6.99
F5	RIPK2	Receptor-interacting serine-threonine kinase 2	-	1.02	1.88	-2.78	-1.91
F6	TNF	Tumor necrosis factor	+	-1.75	-10.14	4.93	25.72
F7	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	+	-1.75	-2.57	76.24	25.02
F8	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	+	1.11	1.70	-1.38	1.14
F9	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	+	1.68	1.01	31.18	11.35
F10	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	+	1.24	-2.07	2.38	2.27

Table 10: PCR Fold Regulations (Cont.)

Well Number	Gene Symbol	Gene Discription	Pro (+) or Anti (-) Apoptotic	U87 3 Day +Cisplatin -Gamma vs 3 Day Control	U87 3 Day +Cisplatin +Gamma vs 3 Day Control	U87 7 Day +Cisplatin -Gamma vs 7 Day Control	U87 7 Day +Cisplatin +Gamma vs 7 Day Control
F11	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	+	-1.03	1.43	-3.86	-3.24
F12	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	+	-1.39	1.40	-1.43	3.28
G1	CD27	CD27 molecule	-	-1.74	-2.68	18.93	97.34
G2	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	+	1.54	-1.17	-2.98	-1.33
G3	TNFRSF10	Tumor necrosis factor (ligand) superfamily, member 10	+	-4.17	-1.76	7.48	4.52
G4	CD70	CD70 molecule	+	1.10	-1.12	-1.51	-1.35
G5	TNFRSF8	Tumor necrosis factor (ligand) superfamily, member 8	+	2.06	2.17	6.42	14.07
G6	TP53	Tumor protein p53	+	1.05	1.42	-3.45	-3.49
G7	TP538P2	Tumor protein p53 binding protein, 2	+	-1.09	1.42	-1.84	-1.26
G8	TP73	Tumor protein p73	+	-1.07	-5.51	49.95	48.67
G9	TRADD	TNFRSF1A-associated via death domain	+	-1.20	1.28	-1.11	1.44
G10	TRAF2	TNF receptor-associated factor 2	-	-1.55	-2.22	1.84	1.54
G11	TRAF3	TNF receptor-associated factor 3	+	-1.18	-1.44	1.14	1.21
G12	TRAF4	TNF receptor-associated factor 4	-	1.05	-1.00	-1.05	-1.09
H01	B2M	Beta-2 microglobulin	N/A	1.32	1.34	-1.19	1.03
H02	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	N/A	-1.01	1.19	-1.75	-1.94
H03	RPL13A	60S ribosomal protein L13a	N/A	-1.13	-2.03	3.42	3.04
H04	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	N/A	-1.16	1.27	-1.64	-1.62
H05	ACTB	Beta-actin	N/A	-1.04	1.42	-3.03	-3.22

Figure 4: U87 3 Day Cisplatin Only Fold Regulation vs. 3 Day Control

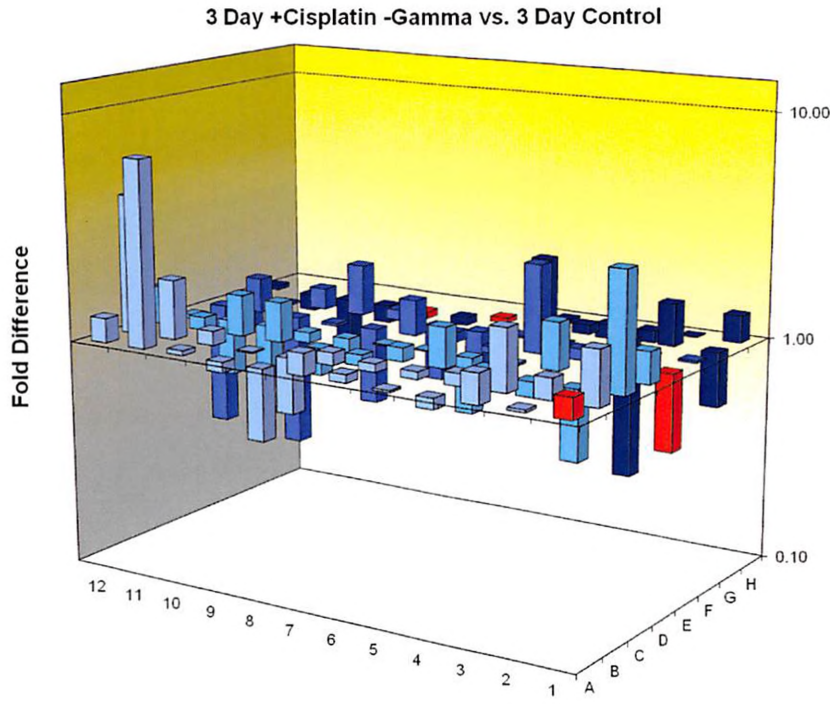


Figure 5: U87 3 Day Cisplatin Plus Gamma Irradiation Fold Regulation vs. 3 Day Control

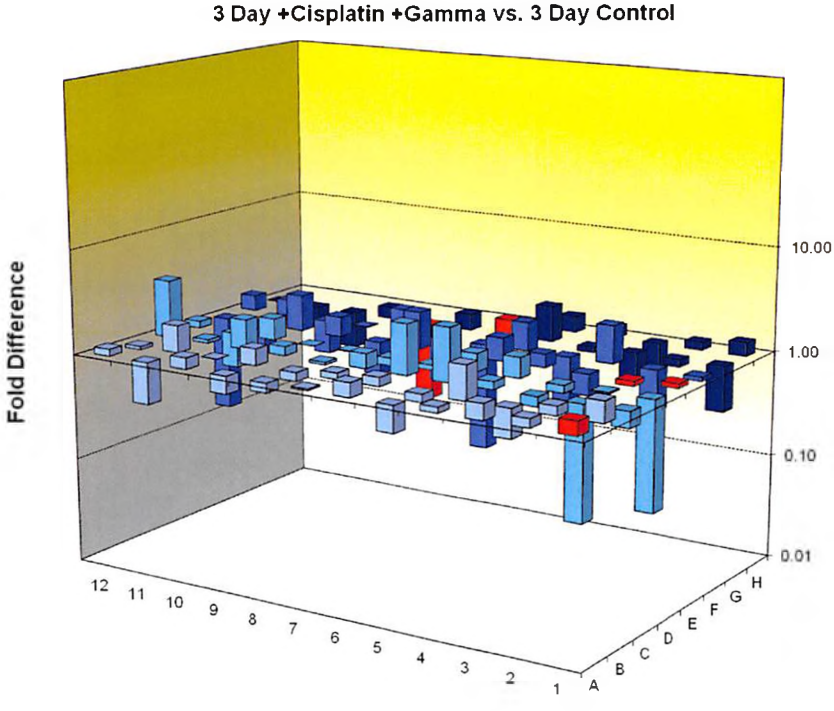


Figure 6: U87 7 Day Cisplatin Only Fold Regulation vs. 7 Day Control

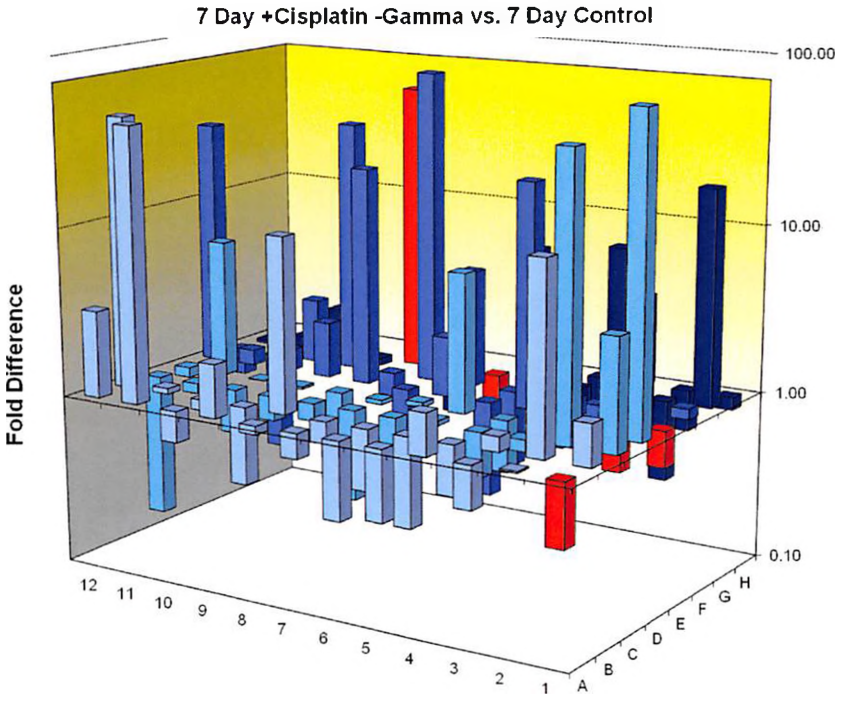


Figure 7: U87 7 Day Cisplatin Plus Gamma Irradiation Fold Regulation vs. 7 Day Control

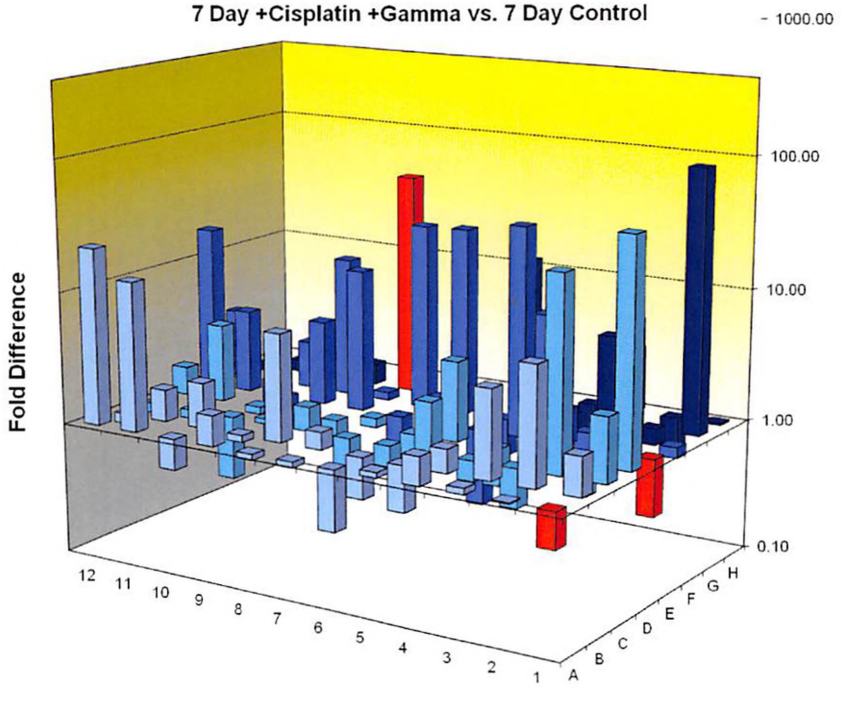


Figure 8: U251 3 Day Cisplatin Only Fold Regulation vs. 3 Day Control

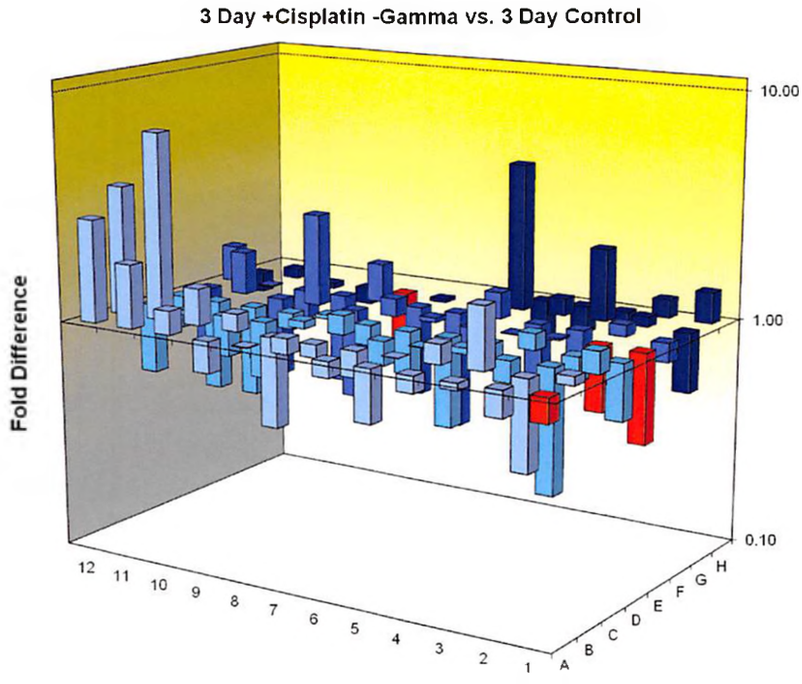


Figure 9: U251 3 Day Cisplatin Plus Gamma Irradiation Fold Regulation vs. 3 Day Control

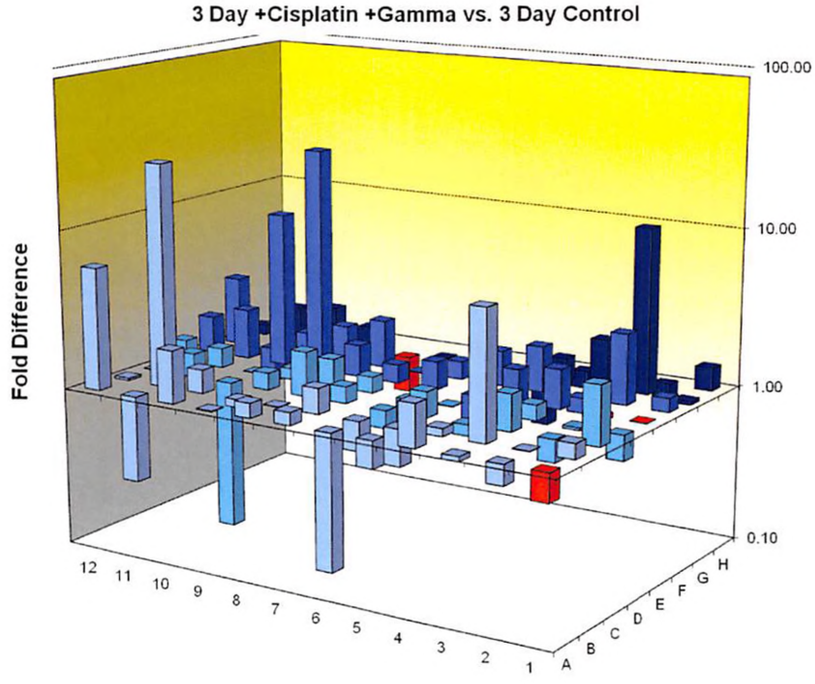


Figure 10: U251 7 Day Cisplatin Only Fold Regulation vs. 7 Day Control

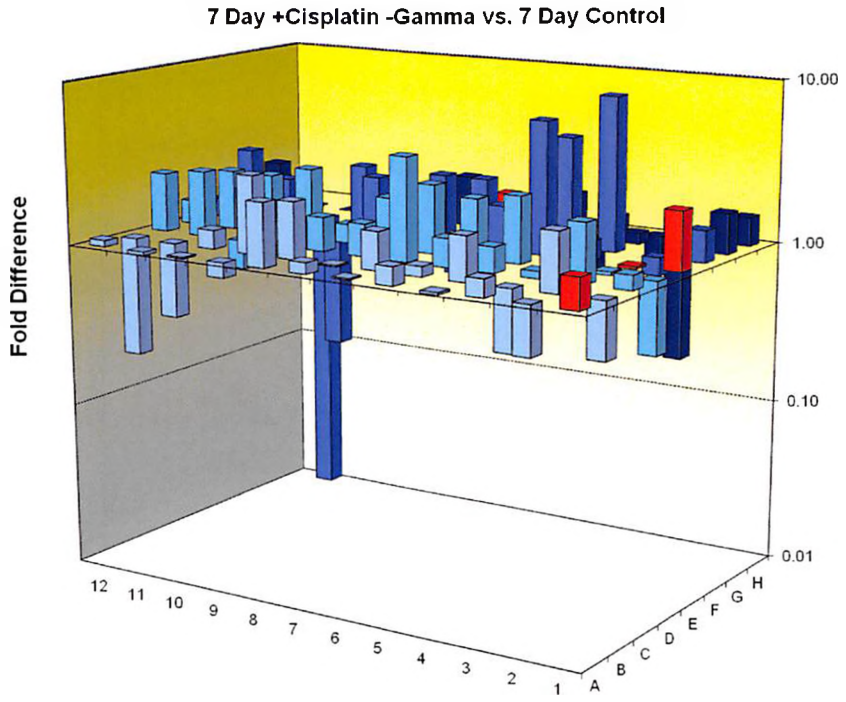
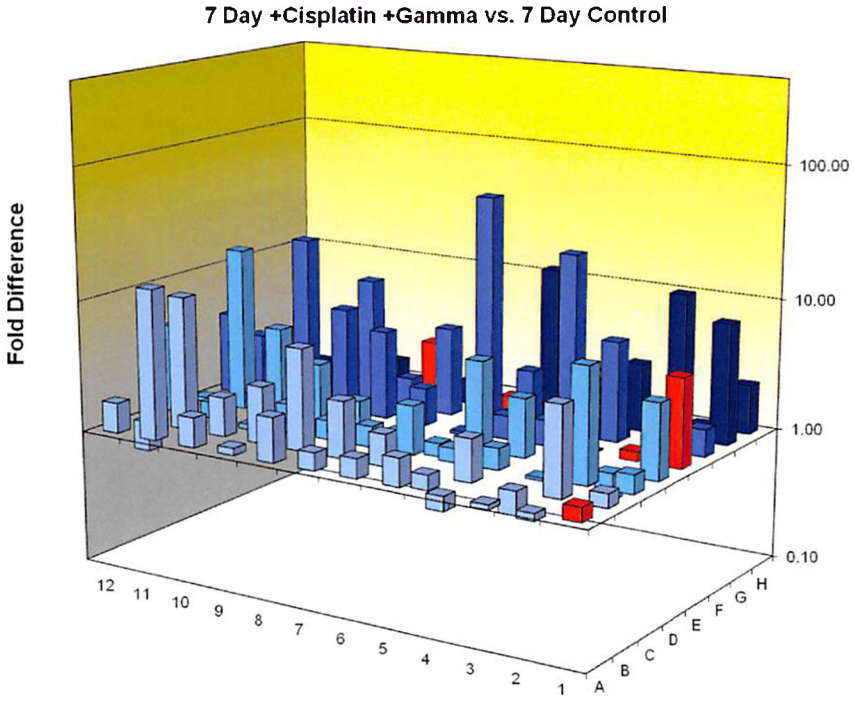


Figure 11: U251 7 Day Cisplatin Plus Gamma Irradiation Fold Regulation vs. 7 Day Control



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